Remarks

In view of the above amendments and the following remarks, reconsideration of the outstanding office action is respectfully requested.

Claims 38-55 have been cancelled without prejudice, and new claims 106-108 have been introduced. Descriptive support for new claims 106 and 107 is provided in the paragraph at page 15, lines 16-27. Descriptive support for new claim 108 is provided at page 20, lines 10-16. Claims 17-37, 56-65, and 106-108 remain pending and under examination. No excess claim fees are due with this submission.

This submission is accompanied by a request for a three-month extension of time (from the deadline of August 28, 2008) and an Information Disclosure Statement (IDS). Fees for the extension of time and IDS have been withdrawn from deposit account 14-1138. Any excess fees can be credited and any fee deficiencies can be charge to this same account.

The rejection of claims 27-37 under 35 U.S.C. § 112, first paragraph, as being enabling only for the treatment of a thrombotic condition, but not for the prevention of a thrombotic condition is respectfully traversed. The data presented in Examples 6 and 8 fully supports the prophylactic method of use as claimed. Because the data demonstrates that platelet activity can be blunted (by pretreatment with a PPARγ agonist), this fully supports the prevention of unwanted platelet activation and, hence, unwanted thrombosis. Therefore, the rejection of claims 27-37 for lack of enablement is improper and should be withdrawn.

The rejection of claims 23-25, 33-35, and 62-65 under 35 U.S.C. § 112, second paragraph, for indefiniteness is respectfully traversed. Claims 23, 33, and 62 recite use of "*an* inducer of a PPARγ agonist" whereas claims 24, 34, and 63 recite that "*the* inducer of a PPARγ agonist" is a particular agent. Thus, claims 23, 33, and 62 provide proper antecedent basis for this language. Moreover, the use of several inducers of a PPARγ agonist are described and claimed in the present application. Therefore, this rejection is improper and should be withdrawn.

The rejection of claims 17-23, 25, 27-33, 35, 37, 56-62, and 64 under 35 U.S.C. § 103(a) for obviousness over the combination of U.S. Patent No. 6,127,394 to Pershadsingh et al. ("Pershadsingh") in view of U.S. Patent No. 7,018,985 to Boyer et al. ("Boyer") is respectfully traversed.

Pershadsingh identifies a class of thiazolidinedione derivatives and indicates that the compounds are activators of PPARγ (col. 11, line 13), although other portions of the reference are unclear whether these compounds are agonists or antagonists of PPARγ (col. 11, lines 13-14). Among a remarkably long list of disparate indications (over 200 diseases or conditions listed in Tables II-VII), Pershadsingh identifies "thrombosis and restenosis after angioplasty" and "myocardial infraction" in Table II. There is, however, no experimental evidence cited in Pershadsingh to support either of these uses, nor is there any reference to prior art support for these therapeutic uses with related compounds (i.e., other thiazolidinediones).

Boyer is cited primarily as background evidence, which the PTO relies on to demonstrate that platelet adhesion and activation were known to be critical events in intravascular thrombosis.

Based on the combination of Pershadsingh and Boyer, the PTO asserts at pages 8-9 of the office action that the treatment of thrombosis as described by Pershadsingh would have involved prevention of platelet activation and aggregation, as taught by Boyer. Applicants respectfully disagree.

Prior to the present invention, it was not known that platelets possessed the nuclear receptor PPARγ. Quite the contrary, persons of skill in the art would have expected that platelets did not possess PPARγ, because platelets lack a nucleus. Given this expectation, persons of skill in the art would have had no basis whatsoever to expect that contacting a platelet with a PPARγ agonist would have any effect at all. Indeed, the present invention involved the identification of a surprising, new non-transcriptional function for PPARγ. The present application demonstrates for the first time that contacting a platelet with a PPARγ agonist can inhibit platelet release of CD40 ligand (as well as thromboxanes and prostaglandins), and also inhibit expression of CD40 ligand on the platelet surface. None of these changes in platelet activity would have been expected by persons of skill in the art given the prior expectation that platelets do not possess the nuclear receptor PPARγ.

The PTO acknowledges at page 8 that Pershadsingh fails even to mention platelets. In fact, the word 'platelet' only appears in the specification of Pershadsingh in the context of conducting blood work to assess a hypothetical patient profile, e.g., "[a]dditionally, a complete blood count, including white cell count and differential, platelet count, and liver function tests...are checked prior to treatment and periodically thereafter" (Example 4, col. 21, lines 4-8). This is mentioned only in the *prospective* examples of

Pershadsingh. And, as noted above, the term "thrombosis" only appears among a rather lengthy list of disparate indications.

Given this expectation and the absence of any teaching whatsoever in Pershadsingh concerning the ability of PPARγ agonist to blunt the activity of platelets, persons of skill in the art would not have expected the contacting of platelets with a PPARγ agonist to be useful in preventing thrombus formation, treating/preventing a thrombotic condition or disease, or preventing platelet aggregation as presently claimed. That platelet activation is involved in thrombus formation—the reason for the PTO's reliance on Boyer—is beside the point. There simply was no expectation in the prior art that the claimed invention could be achieved by contacting platelets with a PPARγ agonist.

Moreover, as further evidence of the nonobviousness of the presently claimed invention, applicants submit herewith two post-filing date references that acknowledge the contribution of the present invention. The underlying research of the present application was published in the research journal *Blood* in 2004 (Akbiyik et al., "Human Bone Marrow Megakaryocytes and Platelets Express PPARg, and PPARg Agonists Blunt Platelet Release of CD40 Ligand and Thromboxanes," *Blood* 104:1361-1368 (2004) ("Akbiyik")). The Akbiyik reference has been cited numerous times (at least 24) by non-inventors, including in reviews by Santilli et al., "CD40/CD40L System and Vascular Disease," *Intern Emerg Med.* 2:256-268 (2007) ("Santilli," copy attached as Exhibit 1) and Borchert et al., "Review of Pleiotropic Effects of Peroxisome Proliferator-Activated Receptor γ Agonists on Platelet Function," *Diabetes Technology & Therapeutics* 9(5):410-420 (2007) ("Borchert," copy attached as Exhibit 2). Santilli recites, at page 262, that the work of the present inventors "identify[ies] in the platelet a *new* target cell for [thiazolidinediones]" (emphasis introduced). Borchert credits the present inventors, with citation to the Akbiyik reference at page 413, as the first to discover that platelets contain large amounts of PPARγ protein.

Because the discovery underlying the present invention represents a paradigm shift in the understanding of platelet function, persons of skill in the art would have recognized at the time the present invention was made—and, indeed, have since—that the platelet represents a new target for PPARγ agonists. As such, persons of ordinary skill in the art would not have expected before October 2003 that contacting mammalian platelets with a PPARγ agonist would "inhibit[] formation of a thrombosis by the mammalian platelets" (claim 17), "inhibit[] platelet activation to treat or prevent the thrombotic condition or disorder" (claim 27), or "inhibit[] aggregation of the mammalian platelets" (claim 56).

For these reasons, the obviousness rejection of claims 17-23, 25, 27-33, 35, 37, 56-62, and 64 over the combination of Pershadsingh in view of Boyer is improper and should be withdrawn.

The rejection of claims 24, 26, 34, 36, 63, and 65 under 35 U.S.C. § 103(a) for obviousness over the combination of Pershadsingh and Boyer, as cited above, further in view of U.S. Patent No. 6,413,931 to Höök et al. ("Höök") is respectfully traversed.

The teachings and deficiencies of the combination of Pershadsingh and Boyer are noted above. Höök describes the use of decorin to bind fibrinogen in the presence of zinc (Zn^{2+}) to prevent fibrin clot formation. The PTO has asserted that one of skill in the art would have used decorin to treat thrombosis given the teachings of Höök. Applicants respectfully disagree for several reasons.

Firstly, Höök fails to overcome the above-noted deficiency of the combination of Pershadsingh and Boyer.

Secondly, the claim language requires "administering ... an inducer of a PPARγ agonist to a mammal in a manner that *provides for said contacting*" (claims 23 and 62) or "administering ... an inducer of a PPARγ agonist to the individual in a manner that *provides for said contacting*" (claim 33), or administering a DNA molecule encoding the inducer, which also is "*effective to cause said contacting*" (as recited in claims 26, 36, and 65). Thus, the use of an inducer of a PPARγ agonist, such as decorin, or a DNA molecule encoding the same, is not for directly acting on fibrinogen as described by Höök, but instead for the entirely different purpose of inducing production of a native PPARγ agonist, which in turn contacts the platelets. Because Höök fails to teach or suggest this new use of decorin or a DNA molecule encoding decorin, the combination of Pershadsingh, Boyer, and Höök is deficient in this regard.

For these reasons, the rejection of claims 24, 26, 34, 36, 63, and 65 for obviousness over the combination of Pershadsingh, Boyer, and Höök is improper and should be withdrawn.

Applicants further submit that new dependent claims 106-108 are patentable for all of the reasons noted above. Moreover, with respect to new claim 108, applicants note that the teachings of Pershadsingh regarding thrombosis concern "thrombosis and restenosis after angioplasty." Because angioplasty is known to cause mechanical trauma to the endothelium, thrombosis and restenosis often occurs; however, this type of mechanical

induction of thrombosis and restenosis is very different from the aberrant thrombosis that is caused by a disease state (*i.e.*, in the absence of mechanical trauma). Therefore, Pershadsingh is also deficient with regard to new dependent claim 108.

In view of all of the foregoing, applicants submit that this case is in condition for allowance and such allowance is earnestly solicited.

Respectfully submitted,

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Exhibit 1: Santilli et al., "CD40/CD40L System and Vascular Disease," *Intern Emerg Med.* 2:256-268 (2007)

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REVIEW

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CD40/CD40L system and vascular disease

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Abstract Several distinct lines of investigation in the context of atherosclerosis dealing with low-grade inflammation, oxidative stress and platelet activation are now emerging, with CD40/CD40L system as the missing link. CD40 ligand is a transmembrane glycoprotein structurally related to tumour necrosis factor-α and more than 95% of the circulating CD40L derives from platelets. CD40L appears as a multiplayer of several cell types in the inflammatory network. The peculiarity of CD40L as an inflammatory mediator derived from platelets expands the functional repertoire of platelets from players of haemostasis and thrombosis to powerful amplifiers of inflammation by promoting the release of cytokines and chemokines, cell activation and cell–cell interactions. The multifunctional role of CD40L, as a simultaneous activator of all these systems, further blurs the intricate relationship between such events both in the physiological systems and the pathological derangement occurring in atherothrombosis.

Keywords sCD40L · Inflammation · Atherosclerosis · Thrombosis

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Introduction

CD40L is gaining much attention for its role in the initiation and progression of atherosclerosis. Several distinct lines of investigation in the context of atherosclerosis dealing with low-grade inflammation, oxidative stress and platelet activation are now emerging, with CD40/CD40L system as the missing link. CD40 ligand (CD40L, CD154) is a 39-kD transmembrane glycoprotein structurally related to tumour necrosis factor-α (TNF-α), originally thought to be restricted to stimulated CD4-positive T cells [1, 2], mast cells and basophils [3]. Subsequent studies demonstrated its expression on platelets, carrying preformed CD40L, which is rapidly translocated onto the cell surface following activation [4]. The receptor for CD40L, CD40, is constitutively expressed on monocyte/macrophages, endothelial cells (ECs), smooth muscle cells (SMCs) [5] and platelets [6]. Studies on the cellular distribution of CD40L indicate that more than 95% of the circulating CD40L derives from platelets [7]. Henn and colleagues have shown that CD40L, cryptic in unstimulated platelets, is expressed on the surface of platelets within seconds of platelet activation and then cleaved to generate a soluble, trimeric fragment, sCD40L [4]. Multiple platelet agonists, including collagen, thrombin and ADP, are able to induce the exposure of sCD40L from platelets [4]. The translocation of sCD40L on the platelet surface requires a few seconds or minutes, similar to the expression of P-selectin, whereas the shedding of the molecule into the circulation is a slower event, lasting 30–45 min depending on the agonist inducing activation [8]. It is now generally accepted that sCD40L is generated by proteolytic cleavage mediated through activation of a membrane-bound protease, possibly a metalloproteinease (MMP), although the involvement of intracellular structures and reactions has been also proposed, i.e., actin polymerisation [9, 10]. In addition, sCD40L may be possibly involved in a self-perpetuating feedback loop, whereby it binds platelet-bound

CD40 leading to further proteolysis of membrane-bound CD40L, with consequent generation of further sCD40L [6].

There are conflicting data as to whether sCD40L stimulates resting platelets by binding to constitutively expressed CD40 during direct cell–cell contact, thus eliciting proinflammatory responses. Inwald et al. demonstrated granule release and enhanced P-selectin expression after incubation of platelets with trimeric sCD40L [11], suggesting that the biological activity of sCD40L may depend on its existence in a biologically active trimeric structure (as in the case of membrane-bound CD40L) [12]. The prothrombotic activity of sCD40L may be attributable to its KGD peptide sequence as infusion of sCD40L with an altered KGD sequence did not reverse the normal phenotype in CD40L-deficient mice [7]. This sequence in turn allows its binding to glycoprotein IIb/IIIa, with consequent stabilisation of arterial thrombi [7]. Proof that sCD40L is a GPIIb/IIIa ligand was obtained through experiments of direct binding of sCD40L to purified glycoprotein IIb/IIIa [7]. More recently, *in vitro* studies showed that GPIIb/IIIa antagonists (eptifibatide, abciximab and tirofiban) are capable of inhibiting the release of sCD40L in a dose-dependent manner [8, 10, 13], although translocation of CD40L from intraplatelet stores to the surface was unmodified [10]. Taken together, these observations strongly implicate CD40L in the triggering and perpetuation of platelet activation.

CD40/CD40L interactions have also been involved in inflammation and thrombosis. In fact, CD40 and CD40L are coexpressed by virtually all of the cells involved in the processes of atherosclerosis at all stages, such as vascular endothelial cells, smooth muscle cells, macrophages, activated T lymphocytes and platelets [14]. CD40/CD40L interaction on these cellular types triggers a series of events occurring in the vascular wall and in the circulation during the ongoing inflammatory response, events which taken together identify the inflammatory and prothrombotic phenotype observed in both the early and late stages of atherosclerosis. Ligation of CD40 on ECS and VSMCs induces the expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1), intercellular cell adhesion molecule-1 (ICAM-1) and P-selectin, which in turn promote the recruitment and extravasation of monocytes and lymphocytes at the site of vascular injury [14]. Further recruitment of lymphocytes is elicited by CD40L-induced secretion of cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) by several cells, thus fostering a predominantly Th1 cytokine-driven immune reaction, characteristic for atherogenesis, and of chemokines, such as macrophage inflammatory protein-1α (MIP-1α), MIP-1β regulated upon activation normal T-cell expressed and secreted (RANTES), stromal derived factor-1 (SDF-1) and monocyte chemoattactant protein-1 (MCP-1) [5]. CD40/CD40L signalling in endothelial cells results in the production of reactive oxygen species (ROS), which antagonise endothelial NO production thus promoting endothelial dysfunction. In addition, this signalling also results in upregulation of the interstitial collagenases MMP-1, MMP-8 and MMP-13, contributing to impairment of plaque stability, and in inhibition of endothelial cell migration, thus preventing reendothelialisation of plaque erosion. Finally, CD40 signalling induces tissue factor (TF) expression [15–17], which, while promoting blood coagulation, is also able to activate platelets, which in turn enhance further CD40L shedding with consequent amplification of the inflammatory reaction. In addition, sCD40L increases stimulation-induced platelet release of ROS through activation of Akt and p38 MAP kinase signalling pathways [18]. Recent *in vitro* and *in vivo* experiments in a mouse model elucidated a novel alternative pathway for CD40L-mediated inflammation, by interaction with the monocyte/macrophage integrin MAC-1. This interaction enhances adhesion and migration of inflammatory cells and myeloperoxidase release *in vitro*, and inhibition of MAC-1 in low-density lipoprotein (LDL) receptor-deficient mice attenuates lesion development and decreases macrophages accumulation [19].

All these events may potentially induce or facilitate an acute thrombotic event (Fig. 1).

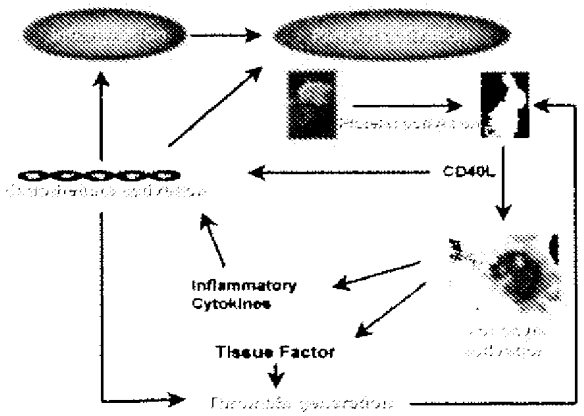

Fig. 1 Role of CD40L in the complex interplay between inflammation, endothelial activation/dysfunction and platelet/coagulative activation. Soluble CD40L, shed from platelets upon platelet activation, triggers monocyte activation, leading to release of inflammatory cytokines and TF expression with thrombin generation, and activation of the endothelium, resulting in further inflammation and platelet activation

CD40L and vascular disease

Coronary artery disease

The pivotal role of platelet activation in atherothrombosis, coupled with the finding that most of sCD40L is derived from platelets, has made CD40L an interesting subject in the setting of cardiovascular disease and of acute coronary syndrome (ACS).

Clinically relevant concentrations of human sCD40L increased the expression of its receptor CD40 in human coronary artery endothelial cells through a mechanism mediated by oxidative stress and extracellular signal-regulated kinase (ERK) 1/2 activation, suggesting a mechanism of amplification of CD40L biological function contributing to endothelial dysfunction and platelet activation in the coronary endothelium [20].

Elevated levels of sCD40L have been reported in patients with both stable or unstable angina (UA) [21], acute myocardial infarction (AMI) [22] and diabetes with angiographically proven coronary artery disease (CAD) [23]. Aukrust and colleagues also performed a series of experiments *ex vivo* to test the hypothesis that the CD40/CD40L dyad may play a pathogenetic role in both the long-term atherosclerotic process and in the triggering of ACS [21]. They also addressed the important issue of the cellular origin of the enhanced sCD40L. In fact, they showed that platelets are able to release large amounts of sCD40L *ex vivo* when stimulated with the thrombin receptor-antagonist peptide SFLLRN in patients and controls. Moreover, increased surface expression of CD40L on T lymphocytes and increased *in vitro* shedding of sCD40L from these cells has also been reported in patients with UA [21]. Thus, T lymphocytes and particularly platelets cause the major contributor to increased circulating sCD40L in the setting of UA. Interestingly, sCD40L-rich serum from patients with UA was able to induce enhanced release of MCP-1 from monocytes, giving rise to the hypothesis that sCD40L may be a contributor rather than the consequence of UA, being responsible for the progressive plaque instability and eventually plaque rupture. On the other hand, procedures such as cardiopulmonary by-pass [24] and percutaneous transluminal coronary angioplasty (PTCA) [25] have been shown to induce sCD40L release, suggesting that elevated sCD40L could be a mere consequence of plaque rupture. To recompose these apparently opposite findings in a unifying hypothesis, it has been speculated that repeated episodes of "minor plaque ruptures" may occur before the onset of clinically manifest coronary event, inducing further elevation of sCD40L, and such a vicious circle may be operating in these patients and may contribute to the progression of the disease.

Some additional information about this issue emerges from the study by Cipollone and coworkers [25] and subsequent studies [26], which demonstrated that preprocedural levels of sCD40L are predictive of enhanced inflammatory response and restenosis after PTCA. A mechanistic basis for this clinical study is provided by the work by Urbich et al., who showed that CD40L inhibits endothelial cell migration by increasing production of ROS [27], thus providing a mechanism by which sCD40L generated by thrombosis could promote restenosis.

A prospective study demonstrated that raised sCD40L is a risk factor for future cardiovascular events in healthy women [5]. Heeschen and coworkers further established the predictive value of this marker by evaluating the clinical relevance of sCD40L in patients presenting with chest pain [22]. In 1088 patients from the c7E3 Fab Antiplatelet Therapy in Unstable Refractory Angina (CAPTURE) Study group, increased sCD40L among patients with chest pain undergoing PTCA identified subjects at risk of death and non-fatal AMI. The increased risk associated with elevated sCD40L was reduced with abciximab pretreatment, a potent platelet glycoprotein IIb/IIIa receptor antagonist. In contrast to troponins, the predictive value of sCD40L levels with respect to the benefit of abciximab is independent of the presence of recent AMI. As a matter of fact, sCD40L identifies the subgroup of patients at highest risk for cardiac events even among those negative for troponin. Whereas positivity of troponins in patients with ACS indicates myocardial necrosis, sCD40L levels reflect the inflammatory thrombotic activity of the culprit lesion in recruiting activating platelets. As the outcome of ACS is highly dependent on the interplay between inflammation and thrombosis, an approach including measurements of these two processes in addition to markers of necrosis may improve our understanding of the underlying pathophysiological processes and allow better assessment of plaque instability.

A subsequent study [28] evaluating plasma sCD40L levels in patients with ACS who eventually reached the study endpoint (death/AMI/congestive heart failure) and in patients who did not, showed significantly higher levels of sCD40L in cases than controls. Patients with the highest sCD40L levels were significantly more likely to develop an AMI or the composite endpoint of death/AMI compared to the patients in the lowest quartiles. Hazard ratios adjusted for troponin I and CRP showed that sCD40L is an independent predictor of the risk of death or MI at 10 months after an ACS.

Recently, genetic polymorphism C807T of platelet glycoprotein Ia has been shown to increase the risk for premature MI and is an independent predictor of sCD40L levels during the acute phase of premature MI and one year after the event [29].

Moreover, in patients with non-ST elevation MI a single nucleotide polymorphism (-3459 A>G) in the CD40L gene is a novel regulator of sCD40L concentrations, and increased sCD40L are predictive of MI and of the efficacy of antithrombotic treatment with dalteparin [30].

These studies do not have the power to establish the causal role of CD40L in the pathogenesis of ACS, but these findings raise the question whether elevated sCD40L levels are a reflection of the pathogenetic role of CD40L in the inflammatory and thrombotic processes or whether they are a mere consequence of the clinical event, namely the result of platelet release after thrombus formation. Even in the latter case of sCD40L elevation as an epiphenomenon, it may constitute a reliable marker of platelet activation and of unstable, prone to rupture plaque. Given

the above findings, it would be worthwhile to evaluate whether lowering the levels of this molecule translates into an improvement of cardiovascular outcomes.

Cerebrovascular disease

Elevated plasma levels of sCD40L were detected in patients undergoing high-resolution magnetic resonance imaging of carotid atheroma with evidence of intraplaque lipid pool [31].

Our group has recently evaluated 42 patients with asymptomatic low-grade carotid stenosis and at least one cardiovascular risk factor [32]. Patients had higher sCD40L as well as C reactive protein (CRP) and IL-6 than controls. Subjects were reviewed annually for a median follow-up of 8 years and 14 patients experienced a cardiovascular event. Cox regression analysis showed that only high sCD40L plasma levels predicted cardiovascular risk, independently of cardiovascular risk factors. Thus, downregulation of this system may represent a potential therapeutic target capable of inducing a more stable carotid plaque phenotype.

Both the ligand and its receptor are overexpressed in human and experimental atherosclerotic plaques, in particular in rupture-prone or ruptured plaques [14].

The CD40L expression on platelets from patients with ischaemic stroke was higher than on platelets from patients with asymptomatic carotid stenosis and from normal subjects [33]. Garlichs and colleagues [34] show CD40/CD40L upregulation in patients with acute cerebral ischaemia. Patients exhibited a significant increase of platelet and T-cell expression of CD40L, as well as of CD40 on monocytes, as compared to controls. Interestingly, CD40L upregulation persisted after 3 months since the initial event. In this light it would be interesting to examine whether sCD40L in the subacute phase may predict the recurrence of ischaemic events [35].

Recently, enhanced sCD40L levels were described in patients with Alzheimer's disease, suggesting a role for CD40L in the pathogenesis of this disease [36]. It is possible to hypothesise that increasing CD40L is a surrogate indicator for the switch from a more stable collagen-rich phenotype to an unstable, rupture-prone phenotype [37], which is ultimately responsible for an acute cerebral ischaemic event or for its recurrence. Whether inhibition of CD40/CD40L dyad may translate into a reversal of such a phenotypic switch remains to be elucidated through intervention studies.

Peripheral artery disease

Recently, the hypothesis of a role for sCD40L in the setting of peripheral artery disease was tested both cross-sectionally and with an intervention study [38]. Plasma sCD40L, in addition to other platelet indices such as soluble P selectin, platelet microparticles and platelet surface expression of CD62 and CD63, is raised in peripheral atherosclerosis and is increased after peripheral artery angioplasty, although levels seem unrelated to clinical severity. sCD40L levels did not correlate with other markers, suggesting that platelets may not be the unique source of sCD40L, and that other cells may contribute to plasma levels.

Pulmonary arterial hypertension

Pulmonary arterial hypertension (PAH) is a complex disease that recognises both inflammation and platelet activation in its pathophysiology [39]. Damas and coworkers have recently reported an upregulation of the CD40/CD40L system even in this setting [40]. Patients with primary and secondary PAH but not those with chronic thromboembolic pulmonary hypertension had increased plasma sCD40L compared to controls. In addition, in the patient group as a whole, a positive correlation was found between sCD40L and prothrombin F1+2, suggesting CD40L and platelet activation associated with the ongoing thrombus formation in this setting. Prostacyclin therapy for 3 months did not affect sCD40L levels, despite clinical benefit. This study suggests that CD40L may play a role in the pathogenesis of PAH, possibly triggering a complex chain of events played by platelets and ECs through chemokine-related mechanisms.

Heart failure

Stumpf et al. [41] have recently shown enhanced expression of CD40L on the platelet surface during chronic heart failure (HF), further supporting enhanced platelet activation in this disorder.

Serum levels of sCD40L were measured in 236 patients with acute HF following MI treated with either angiotensin-converting enzyme (ACE) inhibition or angiotensin II blockade and followed for 2 years, and in 116 patients with chronic HF [42]. Patients with acute HF had increased sCD40L levels, particularly those with severe HF, diabetes or hypertension; when these patients were followed longitudinally, persistently raised sCD40L levels were found throughout the observation period with no effect of captopril or losartan; the increase in sCD40L during follow-up was not seen in patients receiving warfarin therapy; patients with chronic HF also had raised sCD40L, significantly correlated with clinical severity, neurohormonal dysregulation and left ventricular dysfunction; studies from different blood compartments suggest that the vasculature of lower extremities and the failing myocardium itself may produce and secrete sCD40L in chronic HF.

The correlation between sCD40L and LV dysfunction suggests that increased sCD40L levels in HF may not only be a parameter of platelet activation but could also reflect other pathogenic mechanisms in myocardial failure, including activation of MMPs and induction of inflammatory cytokines and chemokines. Whatever the mechanisms, the CD40-expressing cardiomyocytes may directly interact with CD40L, either in its soluble form or expressed on the surface of infiltrating T cells and platelets, contributing to persistent tissue inflammation and remodelling within the failing myocardium. These findings suggesting enhanced release of sCD40L within the coronary circulation further support enhanced CD40/CD40L interaction within the myocardium.

CD40L and cardiovascular risk factors

Diabetes mellitus

The strong correlation between diabetes mellitus (DM) and atherosclerosis suggests that both conditions may share a common background [43]. A clustering of variables related to chronic low-grade inflammation, oxidative stress and platelet activation is emerging as a conceivable "common soil" that may influence the development of both diseases [44].

In vivo platelet activation has been reported previously in diabetes [45, 46] and increased plasma levels of CD40L have been described recently in both type 1 (T1) and type 2 (T2) DM [23, 47]. Moreover, significantly increased co-expression of CD40 and CD40L on platelets of diabetic patients compared with nondiabetic controls has been reported, with a significant correlation of sCD40L with CD40L expression on platelets [48]. Surface expression of collagen receptor Fc receptor-gamma/glycoprotein VI is enhanced on platelets in T2DM and mediates release of CD40L and activation of endothelial cells, thus suggesting enhanced collagen-mediated platelet activation in diabetes contributing to thromboischaemic complications [49].

In T1DM, enhanced sCD40L levels have been detected in patients both with [50] or without micro- and macrovascular complications [51]. Serum CD40L is an independent determinant of intima-media thickness of carotid artery in young Japanese T1DM patients [52]. Patients with T1DM without other cardiovascular risk factors show increased CD40L expression and platelet-monocyte aggregation [53], possibly contributing to the prothrombotic and proinflammatory milieu found in diabetes. In addiction, increased sCD40L levels in patients with T1DM and microangiopathy have been associated with platelet hyperactivity, as assessed by platelet P-selectin expression and soluble P-selectin [50].

The highly significant correlation between plasma CD40L levels and the urinary excretion rate of 11-dehydro-TXB_2, a non-invasive index of *in vivo* platelet activation, supports the likelihood of CD40L release during TXA_2-dependent platelet activation in T2DM [54]. This relation is in keeping with previously reported evidence that CD40L is rapidly upregulated during platelet activation [4] and that platelet CD40 itself provides a mechanism for platelet activation [11]. Diabetics showed significantly reduced plasma CD40L after 7 days of aspirin. In addition, improved metabolic control after a 4-week intensive diabetes programme led to reduction in both sCD40L and 11-dehydro-TXB_2 [54]. The observation that both improved metabolic control and low-dose aspirin, two independent interventions down-regulating platelet activation in this setting, significantly reduced plasma CD40L levels, without any measurable impact on systemic inflammation, as reflected by the non-significant change in CRP plasma levels, strengthens the hypothesis of a contribution of platelets to enhanced CD40L release in T2DM [54]. In addition, the positive correlation observed between enhanced sCD40L and 8-iso$PGF_{2\alpha}$ is in line with the report of increased production of endothelial ROS by CD40L [27], suggesting that in T2DM the release of sCD40L from activated platelets may contribute to increased oxidant stress. Increased lipid peroxidation and persistent platelet activation have previously been reported in patients with T2DM [45, 46]. Thus, on the basis of these findings, a possible vicious cycle may be suggested in which inflammatory stimuli involving CD40L upregulation induce increased lipid peroxidation with consequent platelet activation resulting in further oxidant stress [4] (Fig. 2).

Recently, Lim et al. reported a strong correlation between sCD40L and both IL-6 and TF, supporting a link between the CD40/CD40L system and hypercoagulable

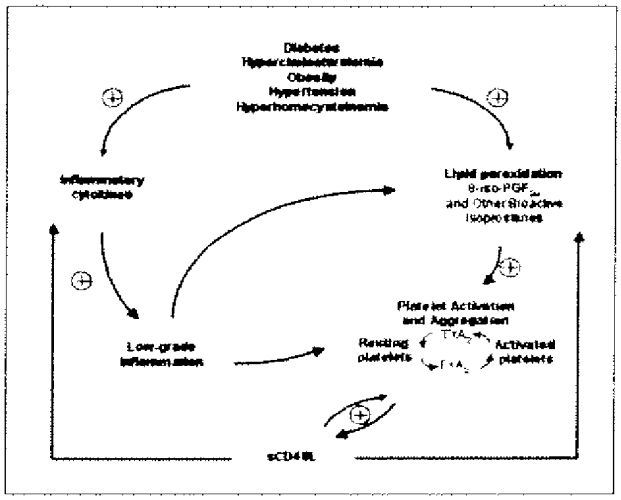

Fig. 2 Role of CD40L in the biochemical mechanisms linking cardiovascular risk factors, inflammation, lipid peroxidation and platelet activation. Potential amplification loops sustaining this chain of events are also shown. *PG*, prostaglandin; *sCD40L*, soluble CD40 ligand; *TX*, thromboxane

state in diabetes [55]. Moreover, elevated plasma sCD40L in patients with diabetes can be reduced by a 1-year multifactorial cardiovascular risk intervention strategy, consisting of ACE inhibitor-based blood pressure control, statin-based lipid lowering, sulphonylurea-based glycaemic control and antiplatelet therapy [55].

The molecular mechanisms linking sCD40L to the accelerated atherosclerosis in diabetes are not yet completely identified. To test the hypothesis that sCD40L is involved in the process of vascular complications in diabetes by triggering a complex group of inflammatory reactions both in vascular endothelial cells and in circulating monocytes/macrophages, we provided evidence in humans that enhanced sCD40L in both T1DM and T2DM is directly responsible for endothelial dysfunction and monocyte activation, demonstrating a positive correlation between sCD40L and ICAM-1, VCAM-1, E-selectin and MCP-1 in diabetic patients [56].

Finally, advanced glycation end-products have been identified as potential triggers of expression and release of sCD40L in diabetic patients [57].

Hypercholesterolaemia

There are several lines of evidence that implicate CD40/CD40L signalling in the vascular pathology associated with hypercholesterolaemia. High cholesterol levels have been associated with enhanced thrombotic risk, possibly related to enhanced persistent platelet activation [58] and thrombin formation [59] observed in this setting. sCD40L is related to cholesterol metabolism as assessed by cholesterol synthesis pathways in patients with moderate hypercholesterolaemia [60]. Patients with moderate hypercholesterolaemia exhibit increased *ex vivo* expression of CD40L and P-selectin on platelets and elevated CD40 expression on monocytes [61]. A larger study [62] showed that in patients with hypercholesterolaemia, increased plasma sCD40L levels have been positively associated with *in vivo* platelet activation, as reflected by plasma P-selectin and urinary 11-dehydro-thromboxane B_2, and with procoagulant state, as reflected by FVIIa and F1+2. Thus, it appears that the CD40/CD40L dyad may provide the mechanistic framework linking inflammation and prothrombotic state in this setting.

A further study was performed to test the hypothesis that CD40L-induced prothrombotic state is mediated by enhancing oxidative stress in the setting of hypercholesterolaemia [63]. Plasma sCD40L, 8-hydroxy-2'-deoxyguanosine, a marker of oxidative stress, and F1+2 levels were significantly higher in patients with hypercholesterolaemia as compared with controls. An additional *in vitro* study showed that CD40L overexpresses TF and increases the thrombin generation rate by an oxidative stress-mediated mechanism that requires activation of NADPH oxidase [64]. This mechanism, in turn, may be responsible for both the accumulation of oxidised LDL within the macrophages, and the prothrombotic state within the plaque. A short-term (3 days) treatment with atorvastatin was able to significantly reduce platelet CD40L and thrombin generation, independently of the lipid-lowering effect of the statin [64].

Cigarette smoking

Cigarette smokers show significantly increased sCD40L levels, surface expression of CD40L on platelets and T cells and of CD40 on monocytes and increased platelet-monocyte aggregates when compared to age- and gender-matched non-smokers [65]. In addition, the surface expression of CD40 on monocytes and of CD40L on platelets correlates with recent intake of nicotine, as assessed by plasma cotinine concentrations and by the number of cigarettes smoked. This finding may represent one of the possible explanations for the increased cardiovascular risk associated with cigarette smoking.

Obesity

We have reported that android obesity is associated with enhanced lipid peroxidation and persistent platelet activation, both improvable by successful weight loss [66]. These abnormalities are driven by inflammatory triggers related to the degree of abdominal adiposity. We found significantly higher plasma CRP levels, in association with enhanced oxidative stress and platelet activation in otherwise healthy women with visceral obesity [66]. Other authors showed increased CD40L levels in patients with severe obesity [67] and that weight loss over a 16-week period of caloric restriction was associated with significant reductions in sCD40L and oxidative stress [68]. In a further study by our group, CD40L plasma levels were elevated in individuals characterised by insulin resistance; however, its relation to increased platelet activation seems to be largely explained by differences in waist-to-hip ratio (WHR) and insulin sensitivity. Successful weight loss over a 12-week period was associated with a statistically significant increase in S_I and decreases in CD40L, CRP and in urinary 11-dehydro-TXB_2 excretion [69].

Arterial hypertension

Recently, 150 patients with different degree of arterial hypertension were evaluated for the expression of the CD40 system. All patients showed a significant increase of CD40 and CD40L coexpression on platelets as well as sCD40L levels compared with controls, with a slight correlation with blood pressure, suggesting that arterial hypertension is in part an inflammatory disorder [70].

Moreover, nondipper hypertensive patients have enhanced plasma CD40L levels as compared to dippers, with CD40L as the main determinant of IMT [71].

In essential hypertensive patients, microalbuminuria, a recognised marker of preclinical atherosclerosis, is not accompanied by enhanced plasma CD40L concentrations in comparison to hypertensive patients with normoalbuminuria [72].

Metabolic syndrome

Metabolic syndrome is gaining recognition as a multiplex cardiovascular risk factor. The presence of metabolic syndrome is independently associated with elevated sCD40L, CRP and coronary disease severity in CAD patients requiring interventional treatment of stable angina [73]. Patients with metabolic syndrome without cardiovascular disease had significantly higher sCD40L and sP-selectin compared with subjects without metabolic syndrome [74]. Moreover, increased sCD40L levels are related to enhanced thrombotic tendency, as shown by the relationship with prothrombin fragment F 1+2 [75].

Therapeutic implications

Thiazolidinediones

Thiazolidinediones, novel insulin-sensitising antidiabetic agents, act as peroxisome proliferator-activated receptor-γ (PPAR-γ) agonists and have been shown to exhibit antinflammatory and antiatherogenic properties [76, 77]. Treatment of diabetic subjects with a thiazolidinedione-type drug results in decreased sCD40L levels [23, 47].

A recent study [78] showed that human bone marrow megakaryocytes and platelets express PPAR-γ, and rosiglitazone is able to prevent thrombin-induced CD40L surface expression and release of CD40L and TXB_2. These results suggest that the reduced plasma levels of CD40L could derive from inhibition of platelet release of CD40L by the dampening effects of the PPAR-γ agonist drug. In this light it is conceivable to think that the antiinflammatory effects of thiazolidinediones may be mediated by modulation of platelet activation, thus identifying in the platelet a new target cell for this class of drugs.

Lipid-lowering drugs

In addition to the well known lipid-lowering effects, statins have been shown to exert anti-inflammatory and antiatherogenic properties [79, 80]. To better address this issue, several authors have investigated the effects of these drugs on inflammatory markers, including CD40L. Atorvastatin, cerivastatin and simvastatin were able to decrease in a dose-dependent manner the constitutive as well as oxidised LDL- or cytokine-induced expression of the receptor/ligand dyad in human vascular endothelial cells, smooth muscle cells and macrophages [81]. Moreover, statins have been shown to decrease CD40 expression and CD40-related activation of vascular cells, effects partially reversed by the HMG-CoA reductase product mevalonate [82, 83]. Both simvastatin and fenofibrate markedly reduced plasma levels of CD40L, as well as CRP, IL-1β, and improved endothelium-dependent vascular reactivity in patients with combined hyperlipidaemia [84]. In a substudy in the Atorvastatin versus Simvastatin on Atherosclerosis Progression (ASAP) trial, sCD40L levels were found to be about 27 times higher in 110 asymptomatic patients with familial hypercholesterolaemia as compared to controls [85]; statin therapy over 2 years markedly downregulated sCD40L levels, regardless of the statin used and with no correlation with the degree of cholesterol lowering. Recently, simvastatin, losartan and combined therapy significantly reduced sCD40L in hypercholesterolaemic hypertensive patients, to the greatest extent in subjects with high baseline CD40L levels [86].

In addition to their role in modulating CD40L in the setting of asymptomatic hypercholesterolaemia [81, 87], statins have proven effective in patients with stable CAD [88] and ACS [89, 90]. In patients with ACS enrolled in the Myocardial Ischemia Reduction with Aggressive Cholesterol Lowering (MIRACL) study [91], atorvastatin started within 24–96 h of admission abrogated the risk of recurrent cardiovascular events associated with high sCD40L over 16 weeks of treatment. Interestingly, atorvastatin did not significantly affect sCD40L concentrations over the treatment period. To explain this paradox, the authors speculate that the target of statins also involves downstream events in the CD40L pathway and signalling; in addition, it involves other inflammatory and thrombotic events marked by elevated sCD40L [91]. Given the detrimental effects of CD40L in the pathogenesis and progression of atherothrombosis, the ability of statins to lower the expression of this molecule may have potential therapeutic implications.

Antioxidants

As stated above, several lines of evidence substantiate a role of oxidative stress in enhancing CD40L expression [81, 92]. In particular CD40L downregulation in the setting of a deficit in gp 91 phox, the central care of NADPH oxidase, unravelled a role for O_2 generated by NADPH oxidase as a trigger of CD40L expression [92]. These interest-

ing data paved the way to studies testing the efficacy of antioxidants as modulators of CD40L release by platelets.

Recently, the adjunct of vitamin C *in vitro* to platelets stimulated with collagen, dose dependently inhibited platelet CD40L expression with no effect on platelet aggregation, and vitamin C infusion (1 g per 45 min) to healthy volunteers was associated with a significant and concurrent decrease of platelet O_2^- and CD40L by approximately 70% [93], independently of agonist-induced platelet aggregation.

In addition, CD40L exerts some of its detrimental effects by enhancing release of ROS in platelets [18] and endothelial cells [27], suggesting an additional potential for antioxidants as modulators of CD40L-induced inflammation and platelet activation. In this light, *in vitro* experiments demonstrated inhibition of CD40L-induced clotting system activation by vitamin C [64].

It would be worth testing additional antioxidants, alone or in combination, to further elucidate the efficacy of this novel therapeutic approach in terms of reduction of both CD40L expression and CD40L-induced detrimental cascade.

Clopidogrel, a potent inhibitor of ADP-induced platelet aggregation, has been reported to block ADP-induced CD40L expression when administered for 7 days to healthy volunteers, leading to the hypothesis that this inhibition may, at least partially, account for the beneficial effects of this drug [95]. Further evidence suggests that clopidogrel lowers levels of CRP and CD40L in patients undergoing PTCA [96, 97]. Patients with stable CAD randomised to 8 weeks of clopidogrel or placebo showed a significant reduction in sCD40L levels in the clopidogrel group [98]. In patients with ACS, a loading dose of clopidogrel (300 mg) attenuated the agonist effects of ADP and thrombin receptor agonist peptide (TRAP) on platelet secretion, aggregation, and formation of platelet-monocyte and platelet-neutrophil conjugates [99]. Plasma levels of soluble CD40L and P-selectin were also significantly reduced and may all contribute to the clinical benefits of the drug in ACS.

A loading dose of clopidogrel followed by 75 mg/day combined with aspirin in patients with UA undergoing PTCA significantly reduced sCD40L levels at 24 h after stenting and during follow-up [100].

Antiplatelet agents

As platelets have been shown to be the main contributors to sCD40 shedding, the most obvious approach to down-regulate this mediator seems to be the use of antiplatelet agents such as aspirin. Neither Aukrust et al. [21] nor Cipollone et al. [25] found any significant association between aspirin use and serum levels of sCD40L. These findings are in agreement with the observation that aspirin administration did not attenuate the increased risk of future cardiovascular events associated with high sCD40L levels in healthy women [94]. Aspirin treatment for 7 days in volunteers resulted in a 50% decrease in the release of sCD40L from collagen-induced platelet aggregates *in vitro*, suggesting that TXA_2 is a necessary costimulator of platelets [8]. Recently, to further characterise the platelet origin of plasma CD40L, the dose and time dependence of the effects of aspirin in T2DM was investigated [54]. A randomised, parallel group dose-response study of three different doses (30, 100, 325 mg/day) of aspirin was performed in 18 T2DM patients. Plasma CD40L was significantly reduced by about 40–50% after 7 days of aspirin treatment, with no apparent dose effect, with a slow pattern of recovery over the 10-day wash-out period. The reduction in plasma sCD40L levels as well as the time course of recovery strongly support the hypothesis that in the setting of T2DM both TXA_2-dependent and -independent mechanisms of platelet activation contribute to enhanced release of sCD40L [54]. Thus, despite aspirin use, more potent platelet inhibitors may be required to inhibit the enhanced release of sCD40L.

GP IIb/IIIa inhibitors

GP IIb/IIIa inhibitors are an important class of drugs used in the management of UA and non-ST elevation MI, as well as in preventing clot formation during PTCA. Because GP IIb/IIIa receptors are a binding site for sCD40L, it has been hypothesised that they might modulate sCD40L release from platelets. Addition of eptifibatide to platelet-rich plasma inhibited sCD40L shedding from activated platelets [8]. Although maximum inhibition of sCD40L release is achieved at clinical dose of these drugs, suboptimal concentration paradoxically potentiated sCD40L release. This finding may help to explain the negative outcome from the orally available GP IIb/IIIa antagonist clinical trials, where suboptimal doses of drugs were typically used.

The aforementioned study by Heeschen and coworkers [22] better defined the clinical relevance of CD40L inhibition by GP IIb/IIIa antagonists, as it was the first to establish a link between sCD40L levels, clinical outcome and the therapeutic tool. A total of 1265 patients with ACS were randomly assigned to receive intravenous abciximab or placebo 18–24 h prior to PTCA. Among patients with the highest sCD40L levels, a significant decrease in the incidence of death or nonfatal MI during the 6-month follow-up was observed in subjects receiving abciximab, as compared to those receiving placebo. In contrast, among patients in the lowest quintiles for sCD40L, no significant benefit was observed with the use of abciximab. Thus, sCD40L levels at baseline may predict both in the short-

and long-term the efficacy of abciximab in improving the rate of cardiovascular events in patients with ACS. The authors did not measure sCD40L levels after abciximab treatment as the trial was not designed to show an effect of the drug on sCD40L plasma levels.

Tirofiban was reported to non-significantly limit the increase in sCD40L after PTCA in patients with stable CAD [101]. Recently, to investigate the effects of abciximab and eptifibatide on sCD40L, 98 ACS patients undergoing PTCA were studied [102]. Eighteen to 24 h after PCI, sCD40L was unchanged in the controls, and reduced 30% in the abciximab-treated group and 9% in the eptifibatide-treated group.

Influence of pre-analytical and analytical factors on sCD40L measurement

Although sCD40L is emerging as a promising marker of thrombotic risk, relatively little attention has been paid to the potential impact of pre-analytical and analytical interferences that may confound interpretation of sCD40L measurements [103–108]. Sampling methods and temperature, in fact, profoundly affect the sCD40L assay [105, 109]. For example, post-harvesting activation of platelets when obtaining serum might account for the higher sCD40L levels found in serum compared to plasma [105]. This finding, together with the close correlation of serum sCD40L with platelet count, supports the possibility that serum sCD40L actually reflects platelet CD40L content [105]. Timing of sample processing in relation to blood collection is another important determinant of sCD40L blood levels [106].

In this light, earlier studies in which serum samples were employed should be interpreted with caution, and the use of serum should be encouraged if the objective is to measure the total pool of sCD40L, including both freely circulating sCD40L and CD40L expressed in platelets, monocytes and T cells [105], but not *in vivo* levels of this cytokine [106]. If this is the case, platelet-depleted citrated plasma samples, processed promptly after collection to minimise *ex vivo* release of sCD40L, should be the preferred sample type [108–110]. Finally, it should be emphasised that sCD40L is actually a pool of free soluble and microparticle-bound (mp-CD40L) forms, the proportion of which is highly variable in each individual [105, 111]. Presently, most protocols used in clinical studies are unable to distinguish either form. Therefore, it is of utmost importance to refine and standardise sCD40L measurement beginning with sample handling techniques such as centrifugation force and time, anticoagulant choice and filtration methods. Once this goal is achieved, we will be able to accurately define the role of sCD40L as a prognostic marker of thrombotic risk.

Concluding remarks

CD40L appears as a multiplayer of several cell types in the inflammatory network.

The peculiarity of CD40L as an inflammatory mediator derived from platelets expands the functional repertoire of platelets from players of haemostasis and thrombosis to powerful amplifiers of inflammation by promoting the release of cytokines and chemokines, cell activation and cell–cell interactions.

Thus, compelling evidence indicating the expression of the CD40/CD40L dyad on many different cell types substantiates the role of this pathway in an extraordinary plethora of biological effects ranging from inflammation, endothelial dysfunction and platelet/coagulative activation. The multifunctional role of CD40L as a simultaneous activator of all these systems further blurs the intricate relationship between such events both in the physiological systems and the pathological derangement occurring in atherothrombosis.

Studies performed so far do not have the power to establish the causal role of CD40L in the pathogenesis of the above-mentioned disease states, but these findings raise the question whether elevated sCD40L levels are a reflection of the pathogenetic role of CD40L in the inflammatory and thrombotic processes or whether they are a mere consequence of the clinical event, namely the result of platelet release after thrombus formation. Even in the latter case, CD40L elevation may constitute a reliable marker of platelet activation and of unstable, prone to rupture plaque, and hopefully, as a predictor of cardiovascular event risk. In this light the availability of an inexpensive, commercially available assay to measure sCD40L levels in several clinical settings may help to identify subjects at major risk of events who may benefit most from more aggressive preventive interventions.

The relationship between sCD40L and global risk assessment remains unclear. In a prospective study increased sCD40L seems to be predictive for future cardiovascular events in healthy women [94]. However, in a large cohort of healthy volunteers, sCD40L levels poorly correlate with both the individual components and the calculated Framingham Coronary Heart Disease Risk Score [112], suggesting the need for long-term follow-up studies to answer the question whether the predictive value of sCD40L is independent of the Framingham based global risk assessment.

Another issue is the association between sCD40L levels, atherosclerotic risk factors and subclinical atherosclerosis. In large and representative multiethnic population studies such as the Dallas Heart studies, sCD40L was not associated with most atherosclerotic risk factors and with subclinical atherosclerosis [113], suggesting that sCD40L is not a reliable tool for screening of early atherosclerosis

in the general population. It is possible to hypothesise that the validity of sCD40L elevation is proportional to the extent of platelet activation in each setting, but this assumption requires further studies.

The key research priorities for the near future will focus on determining the relevance of sCD40L as a putative therapeutic target. Several studies have already shown the ability to lower CD40L levels by several agents, suggesting its potential role as a reliable tool to monitor the efficacy of antiplatelet, antidiabetic or lipid-lowering drugs. Whether lowering the levels of this molecule translates into an improvement of cardiovascular outcomes remains to be elucidated.

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Exhibit 2: Borchert et al., "Review of Pleiotropic Effects of Peroxisome Proliferator-Activated Receptor γ Agonists on Platelet Function," *Diabetes Technology & Therapeutics* 9(5):410-420 (2007)

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Review

Review of the Pleiotropic Effects of Peroxisome Proliferator-Activated Receptor γ Agonists on Platelet Function

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ABSTRACT

The primary target receptor for thiazolidinediones (TZDs) or peroxisome proliferator-activated receptor γ (PPARγ) agonists is a transcription factor in the nucleus of adipocytes and other metabolically active cells, where they improve insulin sensitivity and glucose utilization. TZDs are also able to modify gene expression in macrophages, smooth muscle cells, and endothelial cells. Although PPARγ is considered to be a nuclear receptor, enucleate platelets also highly express this receptor. The aim of this review is to present the current understanding of a direct or indirect effect of TZDs on platelet function. By means of a comprehensive literature search (January 1990–June 2006), publications were obtained that contained specific information about in vitro and in vivo effects of TZDs on platelet function. The effects were studied for different risk biochemical markers, i.e., proteins found to be elevated in the state of procoagulant inflammation and endothelial dysfunction. Improvement of platelet function was reported for all TZDs—troglitazone, pioglitazone, and rosiglitazone. The described effects included reduction of platelet aggregation, suppression of thrombin-induced protein kinase C-α and -β activation, decrease in plasma P-selectin and platelet P-selectin expression, increase in nitric oxide production, inhibition of the Rho/Rho kinase pathway, and inhibition of tissue factor- and platelet-activating factor-induced morphological changes in macrophages. These findings appeared in parallel with reduction of the plasma concentrations of pro-inflammatory risk markers. TZDs seem to have a direct pleiotropic positive influence on platelet function and coagulation and may be helpful in treating the prothrombotic state observed in patients with type 2 diabetes and metabolic syndrome.

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INTRODUCTION

TYPE 2 DIABETES MELLITUS is considered to be one of the major risk factors involved in the development of atherosclerosis. Atherosclerosis is characterized by the infiltration of mononuclear leukocytes into the intima, local expansion of vascular smooth muscle cells (VSMCs), and accumulation of extracellular matrix.[1,2] Its sequelae—ischemic macrovascular events such as coronary artery disease, stroke, transient ischemic attack, and peripheral artery disease[3]—ultimately lead to the high prevalence of macrovascular morbidity and mortality among patients suffering from diabetes (75%).[4,5] The clustering of stand-alone risk factors in diabetic disorders for cardiovascular complications can also be regarded the basis for the high prevalence of microvascular comorbidities such as retinopathy, nephropathy, and neuropathy.[6]

Patients comprising the different metabolic phenotypes observed as the metabolic syndrome must be considered at high risk for atherosclerosis.[7] Recent reports clearly show a prevalent pro-inflammatory state by creating a proliferative and prothrombotic milieu[3,8] in patients with the metabolic syndrome or diabetes.[9] This condition must be considered as the dominating link to atherogenesis as well as the increased rate of cardiovascular events in this population. The metabolic abnormalities encountered in diabetes such as obesity, insulin resistance, hypertension, impaired glucose tolerance (or diabetes), hyperinsulinemia, and dyslipidemia [characterized by elevated concentrations of triglyceride and low-density lipoprotein (LDL), and by low concentrations of high-density lipoprotein], render arteries susceptible to atherosclerosis.[10]

In this context, various reports draw a picture of the vascular state in diabetes with characteristic detrimental alterations such as a dysfunctional endothelium, an impaired coagulation cascade,[11,12] activated and hyperreactive platelets,[11,13] and aberrant fibrinolysis.[14,15] The accumulation of abnormalities in carbohydrate and lipid metabolism is capable of altering the functional properties of multiple cell types, including adipocytes, leukocytes, endothelial cells, and platelets,[2] as well as VSMCs.

Micro- and macrovascular complications are encountered by physicians as the principal cause of death and disability in people with diabetes.[16] Such complications indicate the need for pathophysiologically oriented therapeutic strategies that should not just target treatment of hyperglycemia but also reduce insulin resistance and β-cell dysfunction. The high incidence of diabetes and the devastating reports of the expected future increase into epidemic dimensions drive investigations to explore the relationship of the observed metabolic and cardiovascular disorders as well as to facilitate the selection of an appropriate therapy, as can be observed by numerous scientific publications appearing every month. The thiazolidinediones (TZDs) are potential candidates for this approach because of their multiple beneficial effects on different cell types, their positive influence on the disturbed metabolism of patients with diabetes, and their potential antithrombotic effects. This review aims at providing an overview of the latest consolidated findings about the pleiotropic effects of TZD on platelets and their activation from recent clinical trials.

PLATELETS AND THEIR ACTIVATION

Platelets are essential to hemostasis, and their dysfunction may cause acute vascular events as a consequence of increased thrombus formation. They are anuclear cells derived from megakaryocytes in the bone narrow, but contain small amounts of mRNA and retain the necessary enzymes for mRNA processing.[17] When activated, platelets release a variety of pro-inflammatory hormones and cytokines, such as prostaglandin E_2, transforming growth factor β, thromboxane A_2 (TXA_2), CD40 ligand (CD40L), interleukin (IL)-1β, and plasminogen activator inhibitor-1 (PAI-1).[18] Hemostasis and the pro-inflammatory state are both characterized by a two-phase platelet aggregation, mediated via the membrane-bound glycoprotein complex (glycoprotein IIb/IIIa receptor). Reversible blood clots agglomerate because of augmented platelet adhesion after contact with free collagen fibrils (e.g., following cell wall rupture) or with immune complexes. Platelet-derived prostaglandins induce irreversible

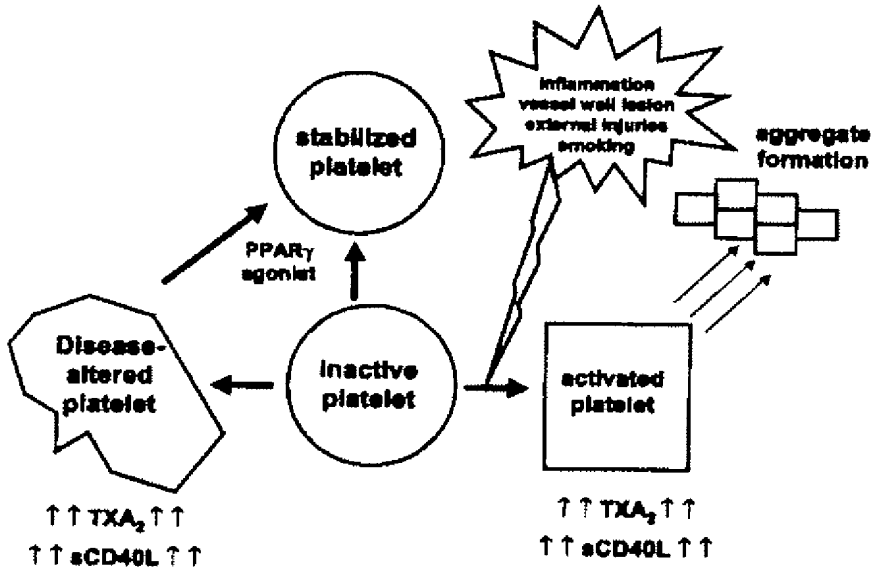

FIG. 1. Cellular effects of PPARγ activation on platelets.

blood coagulation. The final product of the coagulation cascade, fibrin, is generated via thrombin activation in concert with activated platelet factors such as fibrinogen, von Willebrand factor, and several other plasma factors.[19] A graphic representation of the physiologic features of platelets including the cellular effects of peroxisome proliferator-activated receptor γ (PPARγ) activation is provided in Figure 1.

Erosions of the arterial intima may expose subendothelial collagen to blood, which will lead to platelet adhesion and activation. P-selectin expression on platelet surface also facilitates adherence to leukocytes and endothelial cells and enhances the expression of tissue factor on monocytes. Enzymatically shed from platelet surface, soluble P-selectin is considered to be a marker of platelet activation and was shown to be associated with carotid atherosclerosis.[20] Platelets may also be activated directly by platelet-activating factor (PAF).[21] The occurrence of in vivo platelet activation has been reported in type IIa hypercholesterolemic patients, suggesting that high LDL levels, through changes in the composition of platelet membrane phospholipids and cholesterol, may increase platelet reactivity with enhanced TXA_2 biosynthesis.[2] Mildly oxidized LDL has also been reported to induce platelet aggregation through activation of phospholipase A_2 and enhanced TXA_2 secretion from platelets, resulting in release of several potent growth factors.[2] The release of these growth factors, such as platelet-derived growth factor, have mitogenic effects on the fibroblasts in close vicinity to the formed thrombus, and also on the proliferation of other cells in the process of vessel wall remodeling. Mildly oxidized LDL in combination with TXA_2 may also directly stimulate vessel cell proliferation.

The insulin-induced activities include inhibition of platelet aggregation by prompting the synthesis of nitric oxide (NO) in platelets and prostacyclin in endothelial cells. Furthermore, insulin up-regulates prostacyclin receptors and down-regulates α_2-adrenergic receptors in platelets, thereby amplifying the inhibition of platelet aggregation. Insulin also releases tissue plasminogen activator, a potent thrombolytic enzyme, from the platelet membrane, which dissolves the formed thrombus leading to the resumption of normal blood circulation.[22] Platelets from obese subjects and patients with type 2 diabetes are resistant to the ability of insulin or acetylsalicylic acid to counteract

platelet aggregation.[23,24] Under hyperglycemic conditions in insulin resistance or diabetes mellitus, platelets are considered to become hyperreactive.[6] They contribute, in concert with increased cytokine secretion (CD40), to cardiovascular risk as a pro-inflammatory and procoagulant factor. CD40L is normally not expressed on platelet surfaces. In an activated state, however, it is expressed very rapidly. Vascular cell CD40 surface protein interacts with CD40L of platelets and induces the expression of adhesion molecules and the release of inflammatory cytokines (such as IL-6 by monocytes), as well as the procoagulant tissue factor. CD40L is eventually cleaved from the platelet surface and released into circulation as soluble CD40L (sCD40L), which retains its biological activity. Therefore, like platelet-derived soluble P-selectin, sCD40L may reflect platelet activation, and it is considered a potential target biomarker for successful treatment of cardiovascular disease.[25,26]

PLATELET RESPONSE TO PPARγ ACTIVATION: IN VITRO RESULTS

Platelets do not have a nucleus, but it was recently discovered that they contain large amounts of PPARγ protein, while no mRNA for this protein became apparent.[27] The platelet PPARγ protein, which is most likely derived from the parent megakaryocyte, has DNA binding ability that can be enhanced by PPARγ agonists. In vitro, physiological levels of PPARγ agonists can reduce TXA_2 release and expression and release of CD40 ligands from thrombin-activated platelets.[27,28] Pretreatment of human umbilical vein endothelial cells with pioglitazone for 60 min significantly decreased CD40 and CD40L expression induced by oxidized LDL in a concentration-dependent manner.[29]

When thromboxane production was assessed in human erythroleukemia cells and human platelets by means of gas chromatography/selected ion monitoring, the PPARγ agonist troglitazone reduced the TXA_2 production and also reduced thrombin-induced TXA_2 release from platelets in vitro.[30] Activation of the PAF, a key molecule in inflammation, is responsible for cytoskeletal reorganization in macrophages to increase cell motility. Platelets from patients with type 2 diabetes have lost their responsiveness to insulin.[24] As a result, platelets exert increased adhesion, aggregation, and procoagulatory activity after contact with collagen. Pioglitazone inhibits actions of PAF in macrophages by inducing PAF-acetylhydrolase, the enzyme that inactivates the PAF molecule by degradation.[31]

The proliferation of VSMCs is a known response to arterial injury that is an important part of the process of restenosis and atherosclerosis. Troglitazone reduces VSMC migration.[32] The antiproliferative effects of three PPARγ agonists—troglitazone, rosiglitazone, and pioglitazone—on VSMCs derived from the three vascular beds used for coronary artery bypass grafting (the internal mammary and radial artery and saphenous veins) were investigated in vitro by cell counting and cell cycle studies by western blotting for phosphorylated retinoblastoma protein. All three TZDs showed inhibitory potency toward cell proliferation.[33] Table 1 summarizes the results from in vitro studies.

PLATELET RESPONSE TO PPARγ ACTIVATION—RESULTS FROM ANIMAL STUDIES

Next to cell culture experiments, animal studies provided further evidence for a potential antithrombotic effect of PPARγ agonists (Table 2). In a murine model, troglitazone and pioglitazone treatment increased platelet count, decreased platelet-associated antibodies, and increased the survival period of antibody-sensitized erythrocytes.[43] When Sprague-Dawley rats were fed chow mixed with pioglitazone in comparison to placebo, active drug feeding delayed the time to occlusive thrombus formation by 40% without affecting the weight of the thrombus. ADP- as well as arachidonic acid-induced platelet aggregation was inhibited by pioglitazone feeding, which also up-regulated the aortic expression of constitutive NO synthase (cNOS) and thrombomodulin, both of which are considered to be important factors in platelet aggregation and thrombus formation in vivo. These results in-

TABLE 1. EFFECTS OF PPARγ AGONISTS IN REGARD TO PLATELET ACTIVATION AS ASSESSED IN IN VITRO STUDIES

Effect	*Reference (year)*
Platelet	
Reduction of TXA_2	Akbiyik et al.[27] (2004) Hishinumu et al.[30] (2000)
Reduction of sCD40L	Akbiyik et al.[27] (2004) Ray et al.[28] (2006)
Macrophages/monocytes	
Reduction of MCP-1	Zeng et al.[34] (2003)
Reduction of PAF	Sumita et al.[31] (2004)
Fibroblasts	
Reduction of collagen synthesis	Chen et al.[35] (2004) Burgess et al.[36] (2005)
Hepatic stellate cells	
Reduction of collagen synthesis	Galli et al.[37] (2002)
Reduction of MCP-1	Marra et al.[38] (2000)

dicate that pioglitazone administration is able to decrease platelet aggregation and delay intra-arterial thrombus formation in rats.[40] In another animal study, application of pioglitazone in an obese mouse model provided significant protection from thrombosis by prolonging the time to occlusive thrombosis by 40% and 68% at 7 and 15 weeks of age, respectively.[39] Also, following a diet challenge to promote diabetes in the same study, pioglitazone provided protection from occlusive thrombus formation, which was paralleled by significantly lower soluble P-selectin and platelet P-selectin expression, providing evidence of an antiplatelet effect.[39]

In animal models, the amino acid L-arginine has an inhibitory effect on platelet activity.[44] L-Arginine is the natural substrate for NO synthesis,[45] activates fibrinolysis, and inhibits coagulation. L-Arginine administration exerts beneficial effects in conditions associated with endothelial dysfunction and reduced NO synthesis. TZDs are capable of inhibiting the L-arginine degrading enzyme and of the NO-synthesis inhibitor asymmetric dimethylarginine (ADMA),[42] thereby triggering a net increase of serum L-arginine.

Diabetes is associated with enhanced collagen-mediated platelet activation and an increase in extracellular matrix deposition.[46] TZDs decreased extracellular matrix deposition in a rat model of liver fibrosis and inhibited transforming growth factor β-induced collagen and fibrinonectin synthesis in in vitro experiments.[37] PPARγ ligands provide strong apoptotic signals to transformed T cells in concert with the anti-inflammatory mediator prostaglandin D2.[47] In summary, a variety of TZD-induced antithrombotic actions have been reported from various animal models.

TABLE 2. EFFECTS OF PPARγ AGONISTS ON PLATELET ACTIVATION AS ASSESSED IN ANIMAL STUDIES

Effect	*Reference (year)*
Mouse	
Prolongation of time to occlusive thrombus formation	Bodary et al.[39] (2005)
Reduction of P-selectin	Bodary et al.[39] (2005)
Rat	
Prolongation of time to occlusive thrombus formation	Li et al.[40] (2005)
Increase in cNOS expression	Li et al.[40] (2005)
Increase in thrombomodulin	Li et al.[40] (2005)
Reduction of serum homocysteine	Murthy et al.[41] (2005)
Inhibition of ADMA	Wakino et al.[42] (2004)

RESULTS FROM CLINICAL TRIALS

Based on this comprehensive evidence of antithrombotic efficacy of TZDs in vitro and in animal experiments, it was speculated that PPARγ activation may also modulate platelet activation and the release pattern of thrombocyte secretion products in patients with metabolic syndrome and type 2 diabetes. The investigation of the potentially associated clinical effects has just been started in recent years. It was initially shown with troglitazone that PPARγ agonists decrease thrombogenicity by reducing PAI-1 and fibrinogen,[48,49] and the same has meanwhile also been observed with pioglitazone and rosiglitazone. The comprehensive antithrombotic and anti-atherosclerotic efficacy was further demonstrated by reduction of several further biomarkers, including P-selectin, E-selectin, von Willebrand factor, monocyte chemoattractant protein 1 (MCP-1), matrix metalloproteinase (MMP)-9, and high sensitivity C-reactive protein (hsCRP) in numerous controlled clinical trials. An overview on these observations as well as the respective references is provided in Table 3.

The observed positive changes in these biomarkers of platelet function and vascular inflammation were accompanied by improvements in clinically relevant surrogate indicators of cardiovascular risk. In fact, TZDs are the first antidiabetic drug class that has been demonstrated to significantly treat atherosclerosis, which was indicated by a decreased intima–media thickness in several observations.[63–66] This finding was independent from glycemic control[66] and translated into a significant reduction of macrovascular end points by pioglitazone versus placebo in the first appropriately performed large outcome study.[67,68]

IMPLICATIONS OF THE ANTITHROMBOTIC EFFECTS OF TZDs

Insulin exerts antiplatelet activity by stimulating endothelial cell production of the platelet inhibitors prostacyclin and NO, an important mechanism mediating vasodilatation.[69] It was also suggested that pioglitazone administration decreases platelet aggregation and delays intra-arterial thrombus formation in rats, at

TABLE 3. CONFIRMED EFFECTS OF PPARγ AGONISTS ON BIOMARKERS OF PLATELET FUNCTION AND VASCULAR INFLAMMATION AS DERIVED FROM CLINICAL TRIALS

Effect	*Reference (year)*
Reduction of PAI-1	Forseca et al.[48] (1998)
	Kato et al.[49] (2000)
	Kruszynska et al.[50] (2000)
	Derosa et al.[51] (2005)
	Derosa et al.[52] (2005)
Reduction of P-selectin	Sidhu et al.[53] (2004)
	Chu et al.[54] (2005)
Reduction of E-selectin	Sidhu et al.[55] (2003)
	Chu et al.[54] (2005)
	Hetzel et al.[56] (2005)
Reduction of von Willebrand factor	Sidhu et al.[55] (2003)
Reduction of hsCRP	Haffner et al.[57] (2002)
	Sidhu et al.[55] (2003)
	Pfützner et al.[58] (2005)
	Hetzel et al.[56] (2005)
	Pfützner et al.[59] (2006)
	Chu et al.[60] (2006)
	Ghanim et al.[61] (2006)
Reductin of sCD40L	Marx et al.[62] (2003)
	Chu et al.[60] (2006)
Reduction of MMP-9	Haffner et al.[57] (2002)
	Marx et al.[62] (2003)
	Pfützner et al.[58] (2005)
	Chu et al.[60] (2006)
	Ghanim et al.[61] (2006)

least partially, by an increase in the expression of cNOS and thrombomodulin.[40] Under normal conditions, insulin action on platelet function leads to inhibition and dissolution of the newly formed thrombus in the circulation, also by directly suppressing the $P2Y_{12}$ pathway. The platelet $P2Y_{12}$ receptor plays a pivotal role in platelet aggregation, and its activation leads to thrombosis.[70] The $P2Y_{12}$ pathway is up-regulated in patients with type 2 diabetes in comparison to healthy subjects, leading to increased platelet aggregation and increased shear-induced platelet function, which appears to be independent of glycemic control and inflammatory status.[24,71] It is of interest that insulin-treated patients with type 2 diabetes with longer disease duration show reduced effects of the $P2Y_{12}$ antagonist clopidogrel on ADP-induced platelet aggregation as compared with non–insulin-treated patients.[71] The glucose-independent $P2Y_{12}$ up-regulation and the subsequent pro-inflammatory and prothrombotic state that is a characteristic of insulin-treated type 2 patients may be an explanation for the higher rates of restenosis or stent thrombosis that has been observed in this patient population.[72] Therefore, aggressive antithrombotic regimens, which may include novel platelet glycoprotein IIb/IIIa receptor inhibitors, novel $P2Y_{12}$ antagonists, and insulin sensitizers, are recommended for these patients.

Platelets have been identified as key mediators in inflammation. The prevention of release of pro-inflammatory molecules from platelets seems to be a direct effect of TZDs and may have significant implications for the treatment of the increased platelet aggregation and vascular inflammation in patients with diabetes.[28] While part of the antithrombotic activity of PPARγ agonists can be attributed to direct action on the platelets, other TZD effects also indirectly contribute to the observed overall improvement of platelet function (Fig. 2).

TZDs reduce white blood cell count and P-selectin–positive platelets and acute-phase proteins like C-reactive protein, amyloid A, and fibrinogen.[58,73] In addition, TZDs improve endothelial cell function in animals and humans. Pioglitazone administration in streptozotocin-diabetic rats lowered blood pressure, protected

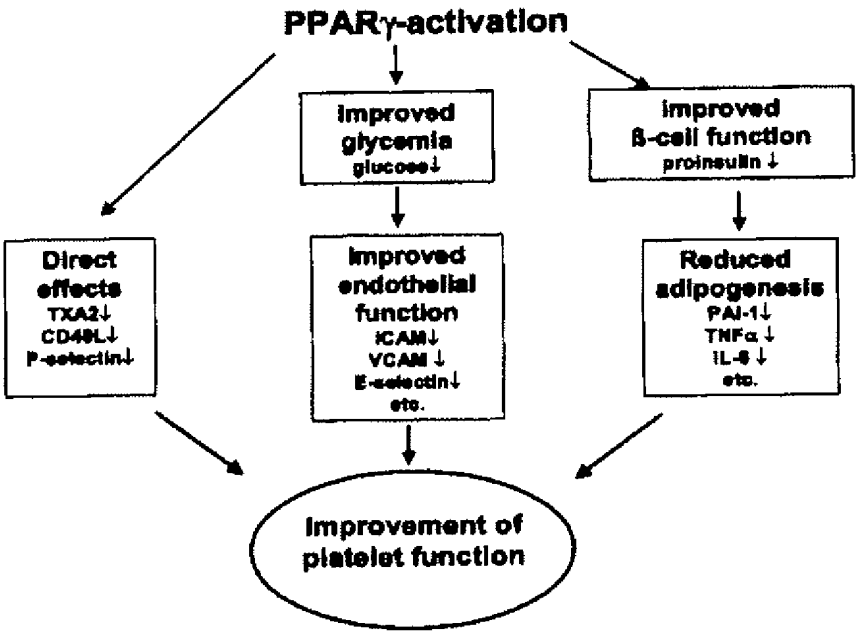

FIG. 2. Factors contributing to the improvement in platelet function by TZD therapy. ICAM, intercellular adhesion molecule; TNFα, tumor necrosis factor-α; VCAM, vascular cell adhesion molecule.

against oxidative stress, and restored endothelial function, which prevented the breakdown of NO and increased NO levels.[74]

High homocysteine levels correlate with progression of vascular disease because of elevation of adverse effects of platelets.[34] Homocysteine up-regulates MCP-1 secretion in monocytes; however, this effect can be prevented by PPARγ activation.[75] The potency of TZD treatment to reduce serum homocysteine levels is controversial: in a rat model, rosiglitazone reduced serum homocysteine levels,[43] whereas this effect was not observed in clinical trials.[76]

In the process of β-cell dysfunction development in type 2 diabetes and metabolic syndrome, exhaustion of the pro-insulin cleavage enzymes in the β-cell leads to increasing secretion of intact pro-insulin, which has been demonstrated to be an independent cardiovascular risk factor.[77] Pro-insulin stimulates adipogenesis resulting in increased secretion of pro-inflammatory markers, such as IL-6, tumor necrosis factor-α, and PAI-1. PAI-1 is the primary inhibitor of endogenous-type fibrinolysis, and its release leads to increased risk of cardiovascular events.[78,79] Treatment of patients with type 2 diabetes with rosiglitazone or pioglitazone has been demonstrated to reduce the levels of circulating intact pro-insulin independent from an improvement of glycemic control, and in parallel with improvement of several other cardiovascular risk factors.[58,59,66] Further supporting effects are the reduction in cytokine expression by several cell types[80] and a decrease in MMP production.[81,82] Elevated MMP-9 and the presence of the described rupture-prone plaques of high lipid composition increase the risk of thrombotic events. In combination with dysfunctional hyperactive and hyperaggregatory platelets of insulin-resistant patients with diabetes, released cell detritus from a ruptured plaque into circulation may well cause ischemic events with fatal outcome. This overall risk of ischemic events can be reduced by TZDs, which has recently been demonstrated with a reduction of stroke, myocardial infarction, and death by 16% over 3 years in the first large cardiovascular outcome trial with pioglitazone, the PROactive Study.[67]

The use of TZDs, exerting their effects through activation of PPARγ as the therapeutic means for glycemic control and anti-inflammatory and antithrombotic effects, represents an important adjunctive approach in the required and necessarily more aggressive antithrombotic therapy in patients with type 2 diabetes mellitus.

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